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The Thermal Stability and Domain Interactions of the Mannitol Permease of *Escherichia coli*

A DIFFERENTIAL SCANNING CALORIMETRY STUDY*

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The thermal stability and domain interactions in the mannitol transporter from *Escherichia coli*, enzyme II^{mtl}, have been studied by differential scanning calorimetry. To this end, the wild type enzyme, IICBA^{mtl}, as well as IICB^{mtl} and IIC^{mtl}, were reconstituted into a dimyristoylphosphatidylcholine lipid bilayer. The changes in the gel to liquid crystalline transition of the lipid indicated that the protein was inserted into the membrane, disturbing a total of approximately 40 lipid molecules/protein molecule. The thermal unfolding profile of EII^{mtl} exhibited three separate transitions, two of which were overlapping, that could be assigned to structural domains in the protein. Treatment with trypsin, resulting in the degradation of the water-soluble part of the enzyme while leaving the binding and translocation capability of the enzyme intact, resulted in a decrease of the T_m and enthalpy of unfolding of the membrane-embedded C domain. This effect was much more apparent in the presence of the substrate but only partly so in the presence of the substrate analog perseitol. These results are consistent with a recently proposed model (Meijberg, W., Schuurman-Wolters, G. K., and Robillard, G. T. (1998) *J. Biol. Chem.* 273, 7949–7946), in which the B domain takes part in the conformational changes during the substrate binding process.

The transport of some carbohydrates from the environment into bacterial cells is mediated by the proteins from the phosphoenolpyruvate-dependent phosphotransferase system (PTS).¹ This system couples the translocation of substrates across the cytoplasmic membrane to concomitant phosphorylation, using phosphoenolpyruvate (PEP) as the energy source. Thus, the overall reaction catalyzed by the PTS system is best described as follows.



REACTION 1

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¹ The abbreviations used are: PTS, phosphoenolpyruvate-dependent phosphotransferase system; DMPC, dimyristoylphosphatidylcholine; DSC, differential scanning calorimetry; EII, enzyme II; HPr, histidine-containing protein; mtl, mannitol; PEP, phosphoenolpyruvate; ptl, perseitol; T_m , melting temperature; PEG, polyethylene glycol.

The equilibrium of this reaction is shifted far to the right.

The transport proteins of the PTS, termed enzyme IIs,² are specific for the carbohydrate they transport but nonetheless show a remarkably similar architecture throughout. In almost all systems, a membrane-spanning domain and two cytoplasmic phosphotransfer domains are found, in some cases covalently bound and in other cases as separate proteins or as combinations of these two possibilities. The phosphoryl group is transferred to the first of the phosphotransfer domains or proteins, the A domain, via two general PTS proteins, enzyme I and HPr, and from there to the second phosphotransfer domain, the B domain. The incoming substrate is bound by the C domain, translocated, and phosphorylated directly by the B domain. Reviews on the PTS systems can be found in Refs. 1–5.

The subject of this study is the mannitol-specific PTS protein from *E. coli*, enzyme II^{mtl}. In this protein, the three domains are all part of the same polypeptide chain, with the C domain at the N terminus and the A domain at the C terminus. The phosphorylation sites are His⁵⁵⁴ on the A domain and Cys³⁸⁴ on the B domain. All three domains as well as the binary combinations, IICB^{mtl} and IIBA^{mtl}, have been subcloned and overexpressed separately and were shown to be enzymatically active in the presence of the other constituents of the PTS (6–10).

In contrast to the large number of studies on the stability of water-soluble proteins, comparatively little is known about the factors determining the stability of membrane proteins in their natural environment. This is mostly caused by the fact that membrane proteins are more difficult to handle than their water-soluble counterparts due to their inherent hydrophobicity, often resulting in aggregation and/or precipitation. The effect is even stronger in the unfolded state, since unfolding results in exposure to the solvent of hydrophobic parts that are normally buried in the interior of the protein. In this study, we circumvented this problem by reconstituting EII^{mtl}, as well as IICB^{mtl} and IIC^{mtl}, in a lipid bilayer, enabling us to study the thermal stability of the enzyme by differential scanning calorimetry and CD spectroscopy.

Data from unfolding studies can also be used to obtain information on the extent of the interactions between the domains in a protein. The energy required to take the enzyme from its

² The nomenclature used is as follows. When referring to domains that are covalently attached, we use the terminology "A domain," "B domain," "C domain," "BA domain," etc. When referring to the domains that have been subcloned and expressed separately, we use the nomenclature IIA^{mtl} for domain A of the mannitol-specific enzyme II, IIC^{mtl} for domain C of the mannitol-specific enzyme II, etc. His-IIC^{mtl} refers to the C domain of enzyme II^{mtl} with a C-terminal His tag of 6 histidines from position 337 in the original sequence. Tryp-IIC^{mtl} refers to the separate C domain of enzyme II^{mtl} obtained from treating the wild type enzyme with trypsin.

It is obvious from what is known about the catalytic mechanism of EII^{mtl} that domain interactions play an important role in the functioning of the enzyme. Therefore, these have been, and still are, the subject of extensive studies (12–14). It was shown that (i) the B and C domains influence each other's conformation as a function of the phosphorylation state of the B domain, (ii) binding of mannitol leads to a large conformational change in the protein, part of which occurs in the B domain, and (iii) in the absence of the C domain there are no extensive stabilizing interactions between the B and A domains in the unphosphorylated or phosphorylated state. In this study, we focus on the B and C domains in order to get better insight into their mutual interactions.

PmaMtlIIChis Plasmid Construction—In order to obtain a construct with a C-terminal His tag, the 51-mer polymerase chain reaction primer 5'-TGCTCTAGATTAGTGATGGTGATGGCTGCTGTTTTTCAGC-AAAATAGC-3' was designed, which was based on the PmaIIc plasmid constructed earlier (9). In this way, the six histidines were positioned directly behind the last predicted membrane-spanning helix at position 337 in the amino acid sequence (19). The *Xba*I site in the primer was used for cloning. A 3'-primer was designed, directed against the beginning of the *MtlA* gene (3'-ATGTCATCCGATATTAAGATCAAA-5'). The *MtlA*-encoding part of PmaMtlA was used as a template for the polymerase chain reaction. Together with the *Pwo* polymerase, this yielded a 1-kilobase pair fragment, which was cloned into the pSK⁺ vector with *EcoRV*. This fragment was then digested with *Nco*I (position 390) and *Xba*I and cloned into the PmaMtlA_{Pr} plasmid, resulting in PmaIIChis-Pr. The entire gene was sequenced and showed the expected sequence.

The enzymatic activity of aliquots of EIIm^{mtl} taken during reconstitution after solubilization in detergent does not give any information on the activity of the protein in the membrane itself. In order to check this, the mannitol phosphorylation and exchange activity of reconstituted EIIm^{mtl} and IICB^{mtl} were determined. The specific phosphorylation activity ranged from 230 to 300 (nmol of mtl-1-P) min⁻¹ (nmol of EIIm^{mtl})⁻¹

in the membranes but increased to approximately 2300 (nmol of mtl-1-P) min⁻¹ (nmol of EII^{mtl})⁻¹ after solubilization of the vesicles in decyl-PEG. Boer *et al.* (9) observed a specific phosphorylation activity of approximately 400 (nmol of mtl-1-P) min⁻¹ (nmol of EII^{mtl})⁻¹ using EII^{mtl} solubilized in decyl-PEG but under slightly different buffer conditions. The specific transphosphorylation activity of EII^{mtl} was in the range 1.3–2.7 (nmol of mtl-1-P) min⁻¹ (nmol of EII^{mtl})⁻¹ at an enzyme concentration of 5 nM and at 30 °C, which compares well with a value of 0.54 (nmol of mtl-1-P) min⁻¹ (nmol of EII^{mtl})⁻¹, determined in the presence of decyl-PEG but also under slightly different buffer conditions (9). The specific transphosphorylation activity of IICB^{mtl} after reconstitution was 0.17–0.35 (nmol of mtl-1-P) min⁻¹ (nmol of IICB^{mtl})⁻¹ at 30 nM enzyme concentration.

The vesicle solutions were further analyzed by electron microscopy, using negative stain to visualize the lipid structures. Mostly multilamellar structures were found that did not disappear after extrusion through polycarbonate filters. Flow dialysis measurements to determine the extent of binding indicated that the vesicles are permeable to small molecules, since no difference in the binding could be detected between reconstituted preparations before and after solubilization in the detergent decyl-PEG (data not shown). It is unlikely that large molecules like enzyme I and HPr are able to permeate the first layer and reach EII^{mtl} molecules inserted in one of the inner layers of a multilamellar vesicle. This would explain the large differences between the effects of detergents on the mannitol exchange and phosphorylation activities, since in the former, only mannitol and mannitol-1-P are needed, whereas in the latter, the transfer of a phosphoryl group from P-HPr to the A domain of EII^{mtl} is the first step. We therefore conclude that the protein has been reconstituted in the lipid bilayer largely in its functional form and that the preparations are suitable for further analysis.

Limited Proteolysis of Reconstituted EII^{mtl} Preparations—Preparations of reconstituted IIC^{mtl} could also be obtained by limited proteolysis of reconstituted EII^{mtl} by trypsin. To 1 ml of freshly prepared EII^{mtl} vesicles, reconstituted using Biobeads in the presence of 100 μM mannitol, 2 ml of reconstitution buffer and 1 ml of a freshly prepared 10 μg/ml solution of trypsin from bovine pancreas were added. This mixture was left at room temperature for 45 min, and then the reaction was quenched by adding 1 ml of a 10 mg/ml solution of soybean trypsin inhibitor (1000-fold excess). Trypsin and soybean trypsin inhibitor were removed by dilution, and subsequent concentration of the vesicle solution by ultracentrifugation as described for mannitol. Samples were analyzed for EII^{mtl}, trypsin and soybean trypsin inhibitor by SDS-polyacrylamide gel electrophoresis. None of these components could be detected on Coomassie-stained gels.

Circular Dichroism Spectroscopy—All CD measurements were performed on an Aviv 62A DS circular dichroism spectrometer equipped with a thermoelectric cell holder for adequate temperature control. Far-UV CD spectra were recorded from 250 to 200 nm, and thermal unfolding between 25 and 95 °C was monitored at 222 nm. In all cases, a 1-mm path length cell and a 1-nm bandwidth was used. The T_m of the protein under study was extracted from the thermal unfolding curves by smoothing the curves and differentiation.

Differential Scanning Calorimetry—All DSC measurements were performed on an MCS differential scanning calorimeter from Microcal (Northampton, MA) with buffer in the reference cell under a 1.5-bar nitrogen pressure. Samples were degassed by stirring gently under vacuum prior to measurements. The gel to liquid crystalline transition of the lipid was scanned between 5 and 40 °C using a scan rate of 30 °C/h and a 2-s filter time. Protein unfolding events were recorded between 40 and 95 °C with a scan rate of 60 °C/h, unless indicated otherwise, and 15-s filter time. In order to check for reversibility of the observed transitions, rescans of the samples were performed after slowly cooling to 30 °C. The scans were analyzed after subtraction of an instrument base line recorded with water in both cells using the software packages ORIGIN (Microcal) or Mathematica (Wolfram Research).

Modeling of DSC Data—A general way to describe an irreversible protein unfolding transition is the model proposed by Lumry and Eyring (25). In this model, an unfolding reaction at thermodynamic equilibrium is followed by an irreversible reaction of the unfolded state, 5521



where *N*, *U*, and *D* are the native, unfolded and denatured states, respectively; *K*_{eq} is the equilibrium constant of the unfolding reaction, and *k* is the first order rate constant of the irreversible step and can be

described by the Arrhenius equation,

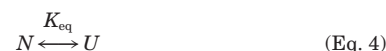
$$k(T) = \exp \left[-\frac{E_a}{R} \left(\frac{1}{T} - \frac{1}{T_k} \right) \right] \quad (\text{Eq. 2})$$

where *E*_a is the activation energy and *T*_k is the temperature at which the rate equals 1 min⁻¹. A detailed theoretical analysis of this model applied to DSC data has been published (26), and in this study it was shown that, under the assumption that the equilibrium is established at all temperatures and that the irreversible step proceeds with a negligible heat effect, the excess heat capacity function could be described by the equation,

$$C_p^{\text{ex}}(T) = \frac{K_{eq}(T)\Delta H}{(K_{eq}(T) + 1)^2} \left(\frac{k(T)}{v} + \frac{\Delta H}{RT^2} \right) \exp \left[-\frac{1}{v} \int_{T_0}^T \frac{k(T)K_{eq}(T)}{K_{eq}(T) + 1} dT \right] \quad (\text{Eq. 3})$$

where *v* is the scanning rate and *T*₀ is a temperature at which both *k* and the concentration *D* are negligible.

Depending on the kinetics of the reversible and irreversible steps, two limiting cases can be defined; the rate of the irreversible step is either 0 or much faster than the rate of the refolding reaction. In the former case, the irreversible step after the unfolding of the protein does not occur, and the unfolding transition is completely reversible. Equation 1 reduces as follows,



and Equation 3 as follows,

$$C_p^{\text{ex}}(T) = \frac{K_{eq}(T)\Delta H^2}{(1 + K_{eq}(T))^2 RT^2} \quad (\text{Eq. 5})$$

which can be used in the deconvolution of DSC data (27). This model predicts that the heat absorption peak in a scan and a rescan of the same sample are equal and that, if the sample is in thermodynamic equilibrium, the result is independent of the scanning rate used in the experiments. Both of these properties can be used to test the model in practice.

In the case of very fast kinetics of the irreversible *versus* the equilibrium reaction, Equation 1 effectively reduces as follows,



since the equilibrium is never established and the intermediate state, *U*, will not be populated at any temperature. It has been shown that the excess heat capacity function in this case can be described by the equation,

$$C_p^{\text{ex}}(T) = \frac{E_a \Delta H}{RT_m^2} \exp \left(\frac{E_a(T - T_m)}{RT_m^2} \right) \exp \left[-\exp \left(\frac{E_a(T - T_m)}{RT_m^2} \right) \right] \quad (\text{Eq. 7})$$

again assuming that the irreversible step proceeds with a negligible heat effect (28). To test whether the model is appropriate, the activation energy can be calculated in four different ways: (a) from a curve fit using Equation 7; (b) from the slope of an Arrhenius plot, where the rates are calculated according to the equation,

$$k(T) = \frac{v C_p^{\text{ex}}(T)}{\Delta H - \Delta H(T)} \quad (\text{Eq. 8})$$

(c) the ratio of the maximum and area of the peak using the equation,

$$E_a = eRT_m^2 \frac{\Delta C_p^{\text{max}}}{\Delta H} \quad (\text{Eq. 9})$$

or (d) the dependence of the temperature of maximum heat absorption on the scanning rate,

$$\ln \left(\frac{v}{T_m^2} \right) = \text{constant} - \frac{E_a}{RT_m} \quad (\text{Eq. 10})$$

For the model to hold true, the activation energies calculated by all four methods should be equal (see Ref. 29 for a detailed discussion).

RESULTS

Reconstitution of EII^{mtl}—Membrane proteins have a pronounced effect on the heat absorption peak of the gel to liquid

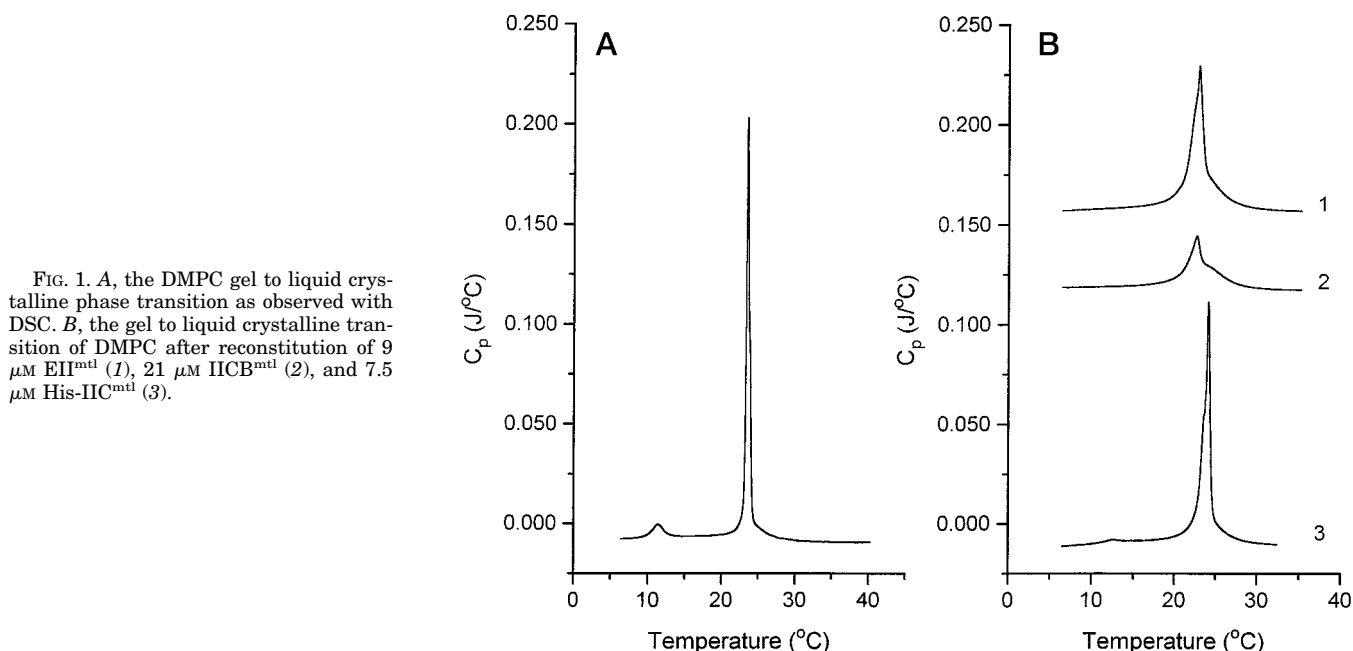


FIG. 1. A, the DMPC gel to liquid crystalline phase transition as observed with DSC. B, the gel to liquid crystalline transition of DMPC after reconstitution of 9 μM EII^{mtl} (1), 21 μM IICB^{mtl} (2), and 7.5 μM His-IIC^{mtl} (3).

crystalline transition of liposomes (30); in general, the T_m shifts downward, the peaks are broadened, and the total enthalpy associated with the phase transition decreases. In Fig. 1, the phase transitions of DMPC vesicles, as monitored by DSC, are shown in the absence of protein (A) and in the presence of 9 μM EII^{mtl} , 21 μM IICB^{mtl} , or 7.5 μM His-IIC^{mtl} (B). In each case, the broadening of the transition is clear, indicating that indeed all three proteins are inserted into the proteoliposomes. Further evidence for insertion into the membrane was obtained from the fact that the broadening effect is dependent on the concentration of the protein (not shown). The peaks observed in the presence of protein can best be described by a broad peak at approximately 23 $^{\circ}\text{C}$ with a sharper peak superimposed. Analysis of these data yielded a linear dependence ($R^2 = 0.99$) of T_m of the sharp transition on the protein:lipid molar ratio, assuming that the final concentrations of the protein and lipid do not differ significantly from the starting situation, whereas the T_m of the broad transition was virtually independent of the protein concentration. The dependence of the lipid phase transition enthalpies on the molar ratio of protein and lipid can be analyzed according to Ref. 31,

$$\frac{\Delta H}{\Delta H^0} = 1 - N_a \left(\frac{P}{L} \right) \quad (\text{Eq. 11})$$

where ΔH^0 is the enthalpy change in the absence of protein, N_a is the number of lipid molecules withdrawn from the phase transition per EII^{mtl} monomer, and P/L is the protein:lipid molar ratio. This analysis yields an estimate of 40 for N_a in the case of IIC^{mtl} .

Thermal Unfolding of EII^{mtl} , Reconstituted in the Presence of 100 μM Mannitol—The thermal unfolding of EII^{mtl} and its separate domains was first studied after reconstitution in the presence of 100 μM of the substrate mannitol. For the wild type enzyme, two transitions could be observed, one centered at about 62 $^{\circ}\text{C}$, the other at about 82 $^{\circ}\text{C}$ (Fig. 2A). In the absence of the A domain, almost all of the first transition disappeared, whereas reconstitution of the C domain led to only one observable transition at 76 $^{\circ}\text{C}$. To ensure that the observed heat absorption peaks were due to structural rearrangements within the protein, the changes of the CD signal at 222 nm, indicative of α -helix content, were also monitored as a function of temperature. The results are shown in Fig. 2B, as well as the

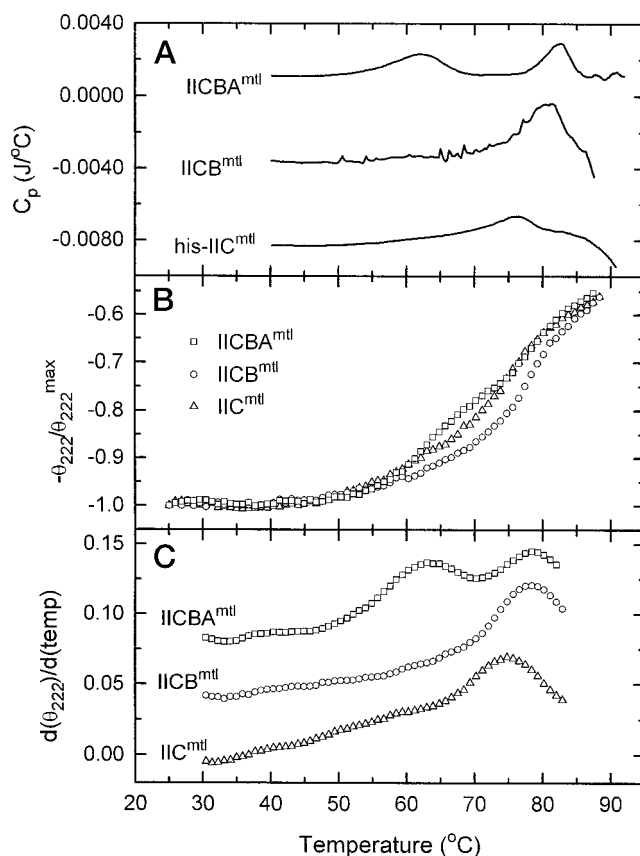


FIG. 2. **Heat-induced unfolding of EII^{mtl} , IICB^{mtl} , and His-IIC^{mtl} , reconstituted in DMPC.** A, raw DSC data. Traces have been displaced along the y axis for clarity. B, change in CD signal at 222 nm, normalized to the signal at 25 $^{\circ}\text{C}$. C, first derivative of the data in B. Experimental conditions were as follows: 50 mM NaP_i , pH 7.5, 10 mM β -mercaptoethanol, 1 mM NaN_3 .

first derivative of these data (Fig. 2C). The T_m values of the high temperature transition observed with CD are somewhat lower than observed with DSC, but this is caused by the slower scan rate in the CD experiments (see below). From the resemblance between the DSC and CD data, it can be concluded that

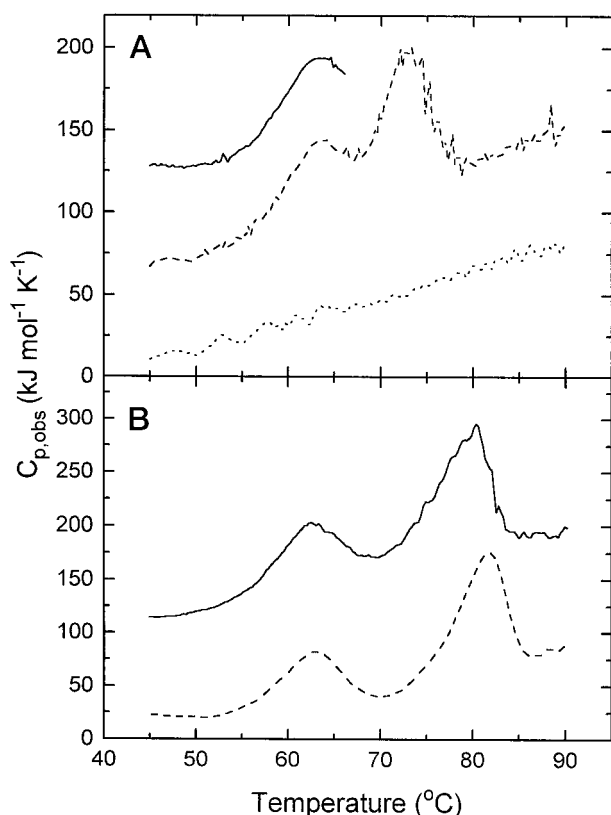


FIG. 3. A, raw DSC data for EII^{mtl} , reconstituted in DMPC. The preparation was scanned to 67 °C (solid line), cooled to 30 °C, and rescanned to 90 °C (dashed line) and again cooled and rescanned (dotted line). No mannitol was present in this preparation. B, scan rate dependence of the thermal unfolding of EII^{mtl} , reconstituted in the presence of 100 μM mannitol. The scan rates were 59 °C/h (solid line) and 120 °C/h (dashed line). Experimental conditions were 50 mM NaP_i, pH 7.5, 10 mM β -mercaptoethanol, 1 mM EDTA, 1 mM NaN₃.

the heat absorption peaks indeed arise from unfolding events in the proteins.

Multiple transitions observed by DSC are usually analyzed using the deconvolution method (27). This method is only applicable to cases where the protein solution is in thermodynamic equilibrium during the complete scan. To satisfy this condition, two criteria must be met; the transition must be reversible (*i.e.* data from a scan and rescans of the same sample should be identical), and the results must be independent of the scanning rate used in the experiment. In our case, no protein unfolding signal could be detected in the rescans after heating the sample to 95 °C (Fig. 3A). However, if the DSC scan was stopped at 67 °C (*i.e.* directly after completion of the first transition), cooled, and rescanned, the first transition was fully reversible. Furthermore, the T_m of this peak was independent of the scanning rate used to record the data (Fig. 3B), and therefore it can be concluded that this peak can be analyzed using a model derived from equilibrium thermodynamics. In contrast, the high temperature peak is both irreversible and dependent on the scanning rate, indicating that the kinetics of the unfolding reaction of this part of the protein also contribute to the shape and the observed T_m of this transition, and therefore this will have to be taken into account in modeling the transition.

Modeling of DSC Data Obtained in the Presence of Mannitol—The data of Fig. 3B were interpreted using Equations 5 and 7 for the low and high temperature transitions, respectively. In total, three independently unfolding units were observed, two in the low temperature region of the spectrum and one in the high temperature region, as indicated by the quality

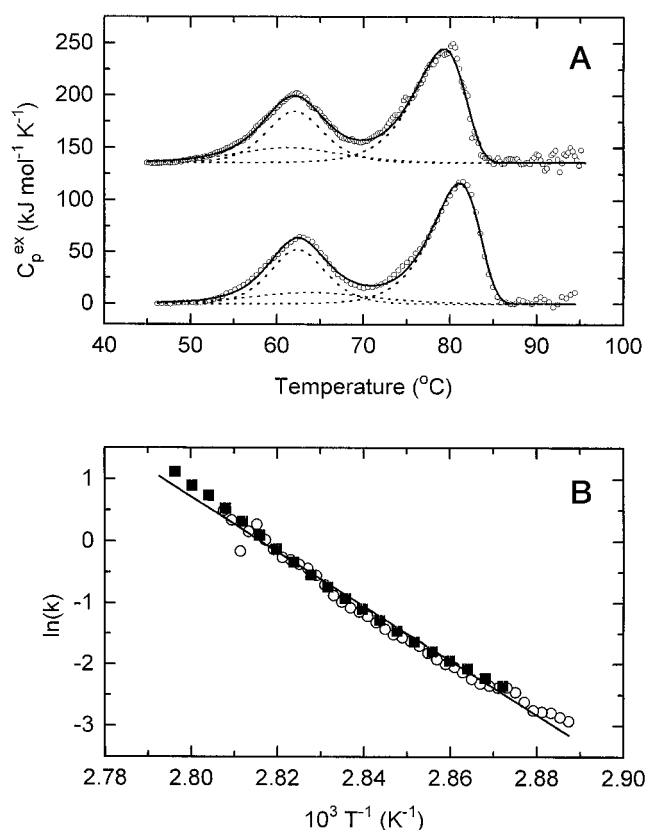


FIG. 4. A, analysis of the thermal unfolding of EII^{mtl} in the presence of mannitol, using the two-state reversible model (Equations 4 and 5) for the low temperature transition and the two-state irreversible model (Equations 6 and 7) for the high temperature transition, as described under "Experimental Procedures." The data are indicated by the open circles, the solid line represents the fit of the data, and the dotted lines show the contribution of each of the individual transitions. The raw data were plotted in Fig. 3B. B, Arrhenius analysis of the high temperature unfolding peak of the data in A. The rates were calculated from Equation 8. The slope of the solid line corresponds to an activation energy of 368 kJ mol⁻¹. Black squares correspond to the data obtained at 120 °C h⁻¹; open circles correspond to the data obtained at 59 °C h⁻¹.

of the fits (Fig. 4A). The estimates for the activation energy of the high temperature transition were 363 and 386 kJ mol⁻¹ at 59 and 120 °C h⁻¹, respectively. To further test the model, an Arrhenius plot was constructed, using Equation 8 for both data sets, resulting in a value of 368 kJ mol⁻¹ for the activation energy (Fig. 4B). The estimates for the activation energy from the height and area of the high temperature peak (Equation 9) and the dependence of T_m on the scanning rate (Equation 10) were 391 and 400 kJ mol⁻¹, respectively, but the latter can, in view of the limited data set, only be regarded as a rough estimate. From the agreement of the values of the activation energy calculated by the four different methods, we conclude that the two-state irreversible model (Equation 6) describes the data for the high temperature transition in the presence of mannitol reasonably well.

Thermal Unfolding of EII^{mtl} in the Absence of Mannitol—In order to study the effect of the substrate on the thermal stability of EII^{mtl} , the unfolding of the enzyme was also studied in the absence of mannitol. To this end, a preparation of EII^{mtl} , reconstituted in the presence of 100 μM mannitol, was washed several times by dilution in a large volume of buffer without mannitol followed by ultracentrifugation. The DSC scan of this preparation is shown in Fig. 5. Removal of the substrate results in a large shift of the high temperature peak from ~80 to 73 °C and a decrease in the total enthalpy of unfolding from approximately 1500 to 1200 kJ mol⁻¹. The unfolding process was

TABLE I
Differential scanning calorimetry of *EII^{mtl}* and separate domains

The data were collected at 60 °C/h unless indicated otherwise and analyzed as described under "Experimental Procedures" and "Results." The S.D. values of the parameters derived from the fits of the data were around 1 °C and 10% for $T_{m,1}$ and ΔH_1 , respectively, 0.2 °C for $T_{m,2}$ and $T_{m,3}$, and 5% for ΔH_2 and ΔH_3 and E_a in the two-state irreversible model. Due to the small contribution of T_k and E_a to the overall shape of the peak in the Lumry-Eyring model compared with the two-state reversible model, these parameters are not well determined from the fits; variations can be as large as 3 °C for T_k and 50% for E_a , and reasonable fits are still obtained. The values in the table for these parameters should therefore be regarded as indicative numbers only. The main source of errors is variations arising from differences in the method of preparation, in the concentration determination, and the choice of the base line that is used to calculate the excess heat capacity function from the raw data. A better estimation of the errors is therefore derived from averaging results obtained for different preparations; these S.D. values are 1.7 °C for $T_{m,1}$, 70 kJ mol⁻¹ (27%) for ΔH_1 , 0.5 °C for $T_{m,2}$, and 60 kJ mol⁻¹ (13%) for ΔH_2 . For $T_{m,3}$ and ΔH_3 , the S.D. values are 1.9 °C and 170 kJ mol⁻¹ (23%) in the presence of mannitol, 1.2 °C and 70 kJ mol⁻¹ (12%) in the absence of mannitol, and 0.8 °C and 120 kJ mol⁻¹ (18%) in the presence of perseitol. The S.D. for E_a in the presence of mannitol is 110 kJ mol⁻¹ (25%).

Protein	Preparation	Preparation method ^a	Model ^b	$T_{m,1}$	ΔH_1	$T_{m,2}$	ΔH_2	$T_{m,3}$	ΔH_3	T_k	E_a
				°C	kJ mol ⁻¹	°C	kJ mol ⁻¹	°C	kJ mol ⁻¹	°C	kJ mol ⁻¹
<i>EII^{mtl}</i>	1	A	I	58.4	390	62.7	550	82.5	711		628
	2	A	I	61.4	230	62.1	427	79.3	837		363
	2	A ^c	I	64.1	202	62.4	444	81.1	858		386
	3	C	R	59.8	196	62.9	431	72.6	552		
			L-E	62.4	200	62.7	421	72.9	502	78.9	272
			I	63.5	177	62.4	423	73.1	523		389
	3	C, +mtl	I	60.1	173	62.4	431	77.6	644		382
	3	C, +ptl	R	61.4	254	63.0	411	72.9	573		
			L-E	62.2	269	63.0	397	73.4	531	79.0	272
			I	63.4	196	62.3	427	73.4	569		381
	3	C ^d	R	60.9	252	63.4	423	72.6	594		
			L-E	62.2	264	63.4	407	72.9	544	78.9	272
			I	61.7	185	62.6	431	73.0	615		377
	4	B	R	60.9	323	63.6	560	70.4	670		
			L-E	61.2	340	63.8	550	70.7	640	78.9	272
			I	61.3	259	63.1	540	70.7	711		408
	4	B, +mtl	I	60.5	289	62.5	477	78.7	933		397
	4	B, +ptl	R	63.9	388	62.7	506	71.7	760		
			L-E	64.9	378	62.5	528	72.0	711	77.8	314
			I	64.2	261	62.6	573	72.1	795		481
	5	C, +mtl	I	60.6	323	61.8	409	81.7	473		556
<i>IICB^{mtl}</i>		A	I	ND ^e	ND			80.6	849		366
<i>His-IIC^{mtl}</i>		A	I					76.2	464		405
<i>Tryp-IIC^{mtl}</i>	3	C	I					72.0	383		523
	3	C, +mtl	I					74.9	405		506
	3	C, +ptl	I					71.9	302		560
<i>IIBA^{mtl}</i>		D	R	59.1	231	64	473				
<i>IIB^{mtl}</i>		D	R	65.3	220						
<i>IIA^{mtl}</i>		D	R			65.1	396				

^a A, reconstituted and measured in the presence of 100 μ M mannitol; B, reconstituted in the absence of mannitol; C, reconstituted in the presence of 100 μ M mannitol, after which the mannitol was removed as described under "Experimental Procedures." D, mixed with preformed DMPC vesicles; +mtl/ptl, 100 μ M mannitol or perseitol added immediately prior to the extrusion step.

^b R, unfolding of the C domain is modeled as a reversible two-state transition; L-R, unfolding of the C domain is modeled according to the Lumry-Eyring model; I, unfolding of the C domain is modeled as an irreversible two-state transition.

^c Scan rate 120 °C/h.

^d Rescan after initial heating to 67 °C (see Fig. 3).

^e ND, not determined.

irreversible, since no reappearance of the heat absorption peaks in rescans of samples after cooling could be observed. Varying the scan rate between 30 and 120 °C/h showed a clear dependence of the temperature of maximum heat absorption for the high temperature transition but did not influence the shape or position of the unfolding transition at 63 °C (data not shown); this is similar to the behavior in the presence of the substrate.

Ligand-free *EII^{mtl}* samples were also obtained by directly reconstituting the enzyme in the absence of the substrate. The results obtained in this way were very similar to those obtained from samples prepared by the washing procedure outlined above (Table I). It can be concluded that the results are independent of the method used to prepare the samples.

Modeling of DSC Data Obtained in the Absence of Mannitol—Deconvolution of the observed excess heat capacity function using the model described above for the data obtained in the presence of mannitol did not result in good fits of the C domain peak (Fig. 5C). Fitting the data with a model that describes the unfolding of the C domain as a transition that is in thermodynamic equilibrium throughout the unfolding pro-

cess, however, showed that the shape of the observed peak deviates only slightly from this at the high temperature side, *i.e.* at temperatures where the rates of the irreversible reaction are the fastest (Fig. 5A). It appears that the two-state reversible model (Equation 4) is an underestimation of the rate of the irreversible reaction, whereas the two-state irreversible model overestimates the reaction rate; therefore, a model that is intermediate between the two extremes seems appropriate. Such a model is the Lumry-Eyring model (Equation 1; Ref. 25). The result of this fitting procedure is shown in Fig. 5B; the values of the parameters obtained with each model are listed in Table I. The variations of T_m and ΔH for the three transitions with the three different models are relatively small, with the largest variation found for the B domain transition. The latter is not surprising, since the enthalpy of this transition is the smallest, and therefore the contribution of this transition to the overall profile is the least pronounced. The analysis outlined above was also applied to data obtained in the presence of the substrate analog perseitol (see below).

Assignment of Unfolding Transitions to Structural Domains—In order to assign the individual transitions observed

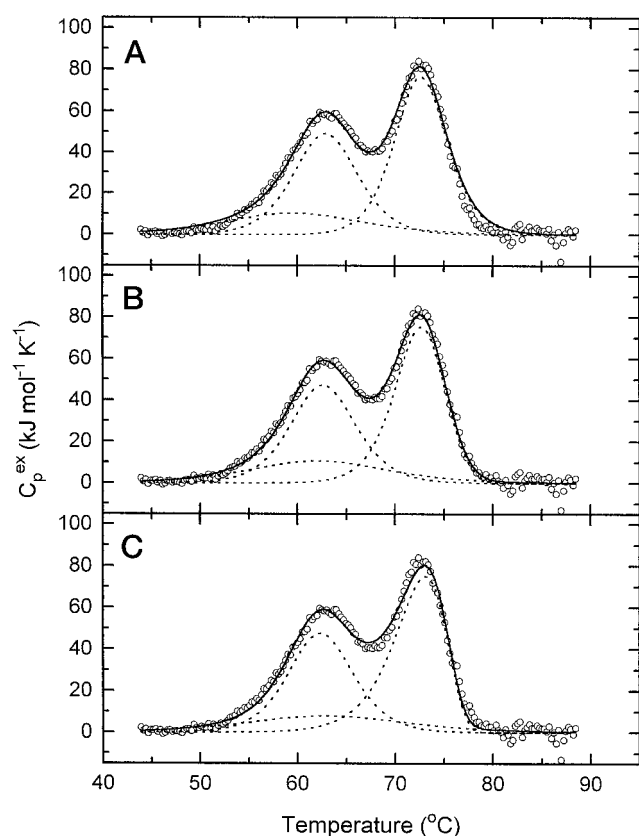


FIG. 5. Deconvolution analysis of the unfolding of EII^{mtl} in the absence of mannitol. The high temperature peak is described by the two-state reversible model (A), the Lumry-Eyring model (B), or the two-state irreversible model (C).

in the unfolding of EII^{mtl} to structural entities in the protein, the unfolding of the separate domains was also investigated and compared with the unfolding pattern of the intact protein. To exclude effects arising from differences between reconstituted preparations, reconstituted IIC^{mtl} was prepared from reconstituted EII^{mtl} by proteolytic cleavage. Exposure of EII^{mtl} to trypsin initially results in the formation of two products of approximately equal size, one of which is water-soluble and has been identified as the binary combination of the B and A domains, whereas the other is embedded in the proteoliposome and corresponds to the C domain. Upon longer exposure to the protease, the B and A domains are fully degraded, but the C domain stays intact, apparently because it is protected from further hydrolysis by the lipid environment. The degradation products and protease can easily be removed by washing the preparation several times with a large volume of buffer and recollecting the proteoliposomes by ultracentrifugation. In this way, reconstituted C domain was obtained that could be directly compared with the intact EII^{mtl} . Stephan and Jacobson (32) showed that tryp-IIC^{mtl}, in their case obtained from inside-out membrane vesicles, is still able to bind and translocate mannitol and that upon complementation with IIBA^{mtl} full phosphorylation and transport activity of the system is restored.

Fig. 6 shows the deconvolution analysis, using Equations 5 and 6, of EII^{mtl} reconstituted in the presence of mannitol (Fig. 6A) as well as the unfolding of tryp-IIC^{mtl} (Fig. 6B) and subcloned IIBA^{mtl} in the presence of DMPC liposomes (Fig. 6C). Unfolding of tryp-IIC^{mtl} results in a single peak at 74.9 °C, the shape of which is close to the shape predicted by Equation 7. For the water-soluble IIBA^{mtl} , two closely separated transitions around 64 °C are observed, arising from the independent unfolding of the B and A domains at 59 and 64 °C, respectively

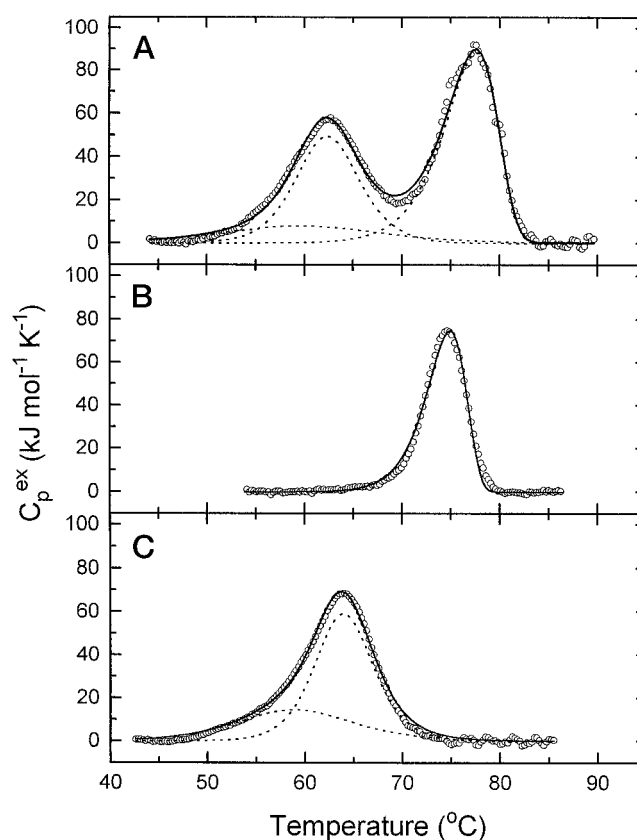


FIG. 6. DSC data after base-line correction and analysis of EII^{mtl} (A), tryp-IIC^{mtl} (B), and IIBA^{mtl} (C). The EII^{mtl} and tryp-IIC^{mtl} data were obtained in the presence of 100 μM mannitol. Experimental conditions were 50 mM NaP_i, pH 7.5, 10 mM β -mercaptoethanol, 1 mM EDTA, 1 mM NaN₃.

(14). Together with the data from Fig. 2, it can be concluded that, when reconstituted in the presence of mannitol, the domains of EII^{mtl} unfold independently; the hydrophilic B and A domains unfold at approximately 63 °C, and the membrane-embedded C domain unfolds between 71 and 82 °C, depending on conditions.

Enzyme-Substrate and Domain Interactions—To gain better insight into the interaction of the enzyme with its substrate, mannitol was added to mannitol-free samples of the native enzyme and to tryp-IIC^{mtl} derived from the same preparation (preparation 3 in Table I). This experiment was repeated using a structural analog of mannitol, perseitol (Fig. 7), a linear C-7 sugar that is bound by EII^{mtl} with a comparable affinity to mannitol (K_d is approximately 100 nM for mannitol and 200 nM for perseitol; Ref. 33) but is not transported or phosphorylated (34). The addition of mannitol to the wild type enzyme led to an increase of the T_m of the C domain from 73 to 78 °C with a concomitant increase in the enthalpy of unfolding of approximately 125 kJ mol⁻¹, depending on the model used to interpret the data; the unfolding transitions of the B and A domains remain largely unaffected. The addition of perseitol has a much smaller effect on the unfolding behavior; the T_m of the C domain increases ~ 1 °C, and ΔH increases approximately 40 kJ mol⁻¹ (Fig. 8). These changes are reflected in the results obtained for the reconstituted tryp-IIC^{mtl}, obtained from the same preparation. The removal of the A and B domains by trypsin results in a decrease of 1 °C for T_m and 120 kJ mol⁻¹ for the enthalpy of unfolding of the C domain. Upon the addition of mannitol, both T_m and ΔH increase but remain smaller than observed for the C domain in intact EII^{mtl} under the same conditions. The addition of perseitol has little effect on the T_m

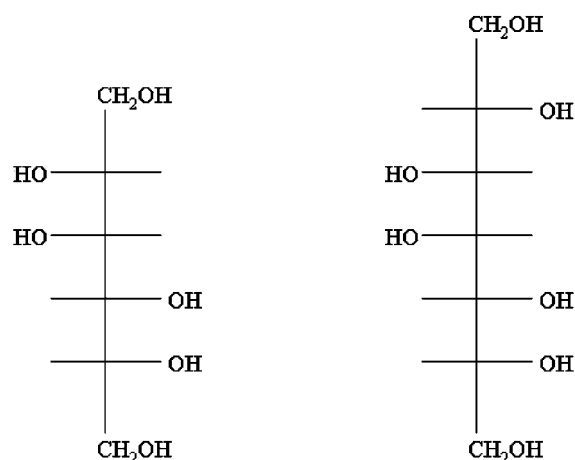


FIG. 7. **Mannitol (left) and perseitol (right).** Both molecules are shown in Fischer projection.

of tryp-IIC^{mtl}, but ΔH appears to be smaller than in the absence of ligand.

DISCUSSION

Reconstitution of EI^{mtl} —In the reconstitution process, three factors play a role: the protein, the lipids, and the detergent. We have chosen DMPC for two important reasons: (a) the protein was shown to be stable when inserted in membranes composed of this lipid³ and (b) the gel to liquid crystalline phase transition of the lipid bilayer can be monitored to obtain information on the reconstitution process without affecting the protein. We chose the detergent *n*-decyl- β -D-maltoside despite its 2 mM critical micel concentration because, unlike for example decyl-PEG, which is used during the purification, it is a homogeneous preparation, and, more importantly, EI^{mtl} is stable and exhibits a good activity in this detergent.

The insertion of EI^{mtl} , IICB^{mtl}, and IIC^{mtl} into the lipid bilayer of DMPC vesicles leads to a decrease in T_m of the gel to liquid crystalline phase transition, the development of a second component to the heat absorption peak, and a general broadening of the transition. In the case of IIC^{mtl}, approximately 40 lipid molecules per inserted membrane protein molecule are withdrawn from the cooperative phase transition. The same analysis applied to DMPC/bacteriorhodopsin systems yields values of approximately 20–40 (30). Since the membrane parts of EI^{mtl} and bacteriorhodopsin are of similar size, a comparable number of lipid molecules can be expected to bind to these proteins when inserted into the membrane. The fact that this is indeed observed and the fact that both proteins are enzymatically active support our conclusion that EI^{mtl} is inserted in a way that is comparable with its physiological state.

The Assignment of Unfolding Transitions to Structural Domains—A critical step in the analysis of the data is the assignment of the unfolding events to structural entities in the protein. Although each of the three separate domains undergoes an unfolding transition between 40 and 95 °C, we observed only two peaks in this temperature interval. The removal of the A domain from the intact protein leads to a large decrease of the transition at 62 °C (Fig. 2), and on the basis of this result it can be concluded that this transition is, at least partly, due to the unfolding of the A domain. Furthermore, the T_m of this transition is independent of the amount of mannitol or perseitol present, as is to be expected for a part of the protein that is far removed from the mannitol binding site. A very small transition remains around 60 °C that disappears if the B domain is

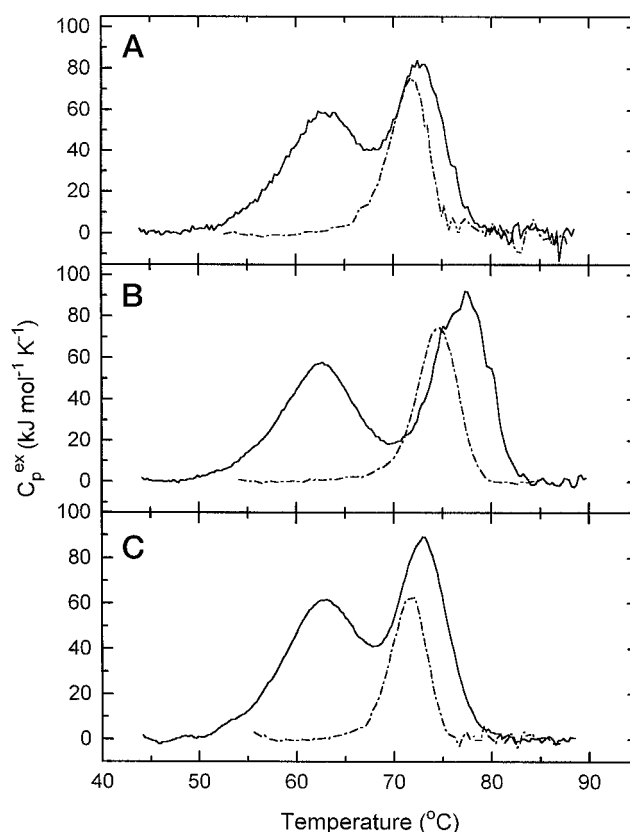


FIG. 8. DSC data after base-line correction of EI^{mtl} (solid line) and tryp-IIC^{mtl} (dotted line), obtained from the same preparation, in the absence of mannitol or perseitol (A), in the presence of 100 μM mannitol (B), and in the presence of 100 μM perseitol (C). Experimental conditions were 50 mM NaP_i, pH 7.5, 10 mM β -mercaptoethanol, 1 mM EDTA, 1 mM NaN₃.

removed, indicating that this transition originates in the B domain. Further evidence for the assignment of the first transition to both B and A domain unfolding comes from the striking resemblance of the deconvolution of the unfolding profiles of IIBA^{mtl} and the wild type enzyme (Fig. 6). The high temperature transition for His-IIC^{mtl} at 76 °C is assigned to the unfolding of the C domain. Removal of the B domain in the presence of mannitol leads to a decrease in melting temperature and a smaller width at half-height for this transition, which is most convincingly seen in the comparison of samples before and after treatment with trypsin (Fig. 8). This is discussed in detail below.

Modeling of the DSC Data—The unfolding of the B and A domains can be described by standard equations, derived from equilibrium thermodynamics, since the transitions are reversible and independent of the scanning rate. The unfolding of the C domain, however, is kinetically controlled (both in the presence and absence of the ligand), and therefore a model that specifically takes this into account is the appropriate choice in a deconvolution type analysis. In the presence of the substrate mannitol, the data can be described well by the simple two-state irreversible model of Equation 2, as shown under “Results.” This model does not take into account effects of dissociation of ligands or the monomerization of the EI^{mtl} dimer upon unfolding. The fact that the data can be described by this model suggests that these events either do not take place or only happen after the rate determining step in the unfolding reaction has taken place. From our data, we cannot discriminate between these two possibilities, and therefore we have chosen the simplest model that accurately describes the data.

³ G. Perkins and G. K. Schuurman-Wolters, unpublished results.

In the absence of the substrate, the simple two-state irreversible model no longer holds, and a more complicated model has to be invoked to describe the data. We have used the Lumry-Eyring model in this case, since it can be regarded as intermediate between the two-state equilibrium and irreversible models. We do not have evidence other than the goodness of fit that this is appropriate, because the analysis of scanning rate-dependent data in the absence of mannitol was hampered by conformational heterogeneity of the C domain, obscuring the shape and T_m of the main peak. However, the values for the thermodynamic parameters obtained from an analysis assuming a two-state reversible model, a two-state irreversible model, or the Lumry-Eyring model did not vary much; the largest variations are found for the transition characterized by the smallest enthalpy of unfolding (B domain). This makes us confident that the basic assumption of our analysis, the existence of three independent unfolding transitions, is correct and that the parameters determined are reasonably accurate.

The analysis of the DSC data in this work yields values for T_m and ΔH for the water-soluble A and B domain in the wild type enzyme. The unfolding of IIA^{mtl}, IIB^{mtl}, and IIBA^{mtl} has been studied previously in detail but under slightly different buffer conditions (14). The results obtained here are, within the error of the experiment, in agreement with those data, indicating that the presence of the DMPC vesicles does not influence the stability of the A and B domains in the absence of the C domain. Also the addition of mannitol to IIB^{mtl} in the absence of the C domain does not alter the stability of this protein (data not shown), and therefore changes in the stability or conformation of the enzyme upon the addition of mannitol are all mediated through the C domain.

The Thermal Stability of EII^{mtl}—The enthalpy of unfolding of the C domain in EII^{mtl} in the absence of ligands, averaged over all estimates in Table I is $600 \pm 70 \text{ kJ mol}^{-1}$ or $18 \pm 3 \text{ J g}^{-1}$. This value is low compared with the average value of 33 J g^{-1} for water-soluble proteins (35) but higher than the values obtained for other membrane proteins such as cytochrome C oxidase from beef heart, yeast, and *Paracoccus denitrificans* (11.3, 10, and 12 J g^{-1} , respectively; Refs. 36–39) or bacteriorhodopsin ($13\text{--}16 \text{ J g}^{-1}$, depending on pH; Ref. 40). The low values of the enthalpy of unfolding compared with water-soluble proteins have been attributed to the higher stability of the membrane-embedded parts. The stability of these parts is so high that they do not completely denature but only partially unfold, leaving large parts of the structure intact, thus contributing only moderately to the total enthalpy of unfolding. In the case of EII^{mtl}, this is reflected in the change of the CD signal at 222 nm, the wavelength specific for α -helices. Almost 60% of the signal at 30 °C is still present after heating to 95 °C (Fig. 2), indicating that a large part of the putative six transmembrane helices (19) is still intact after heat denaturation. However, these structures only account for approximately 50% of the total mass of the C domain; the remainder may protrude into the aqueous phase. It is likely that this is the part of the protein that is responsible for the observed unfolding transition, explaining the somewhat higher enthalpy of unfolding compared with other membrane proteins.

Domain Interactions in EII^{mtl}—The enthalpy of unfolding of the transition assigned to the B domain in EII^{mtl} is also small, $18 \pm 5 \text{ J g}^{-1}$ averaged over the entire data set of Table I. This small value was also reported in an earlier paper on the interactions between the A and B domains (14), where it was attributed either to the high flexibility of the B domain necessary for the domain to be able fulfill its phosphoryl group transfer function or to missing B/C domain interactions. Of these two possibilities, the latter now seems the more likely. Fig. 8 shows

the raw DSC data for the unfolding of EII^{mtl} and tryp-IIC^{mtl}, obtained from the same preparation of reconstituted enzyme. This type of comparison ensures that only those differences arising from the removal of the A and B domains will be observed. In each case, the enthalpy of unfolding of the C domain in the absence of the B domain is lower than when the B domain is covalently attached, most notably so when the substrate mannitol is bound. In the latter case, the T_m of the C domain in the wild type enzyme is $\sim 2.7^\circ\text{C}$ higher, and ΔH is $\sim 200 \text{ kJ mol}^{-1}$ larger than in the case of tryp-IIC^{mtl} (Fig. 8 and Table I). This suggests that an interaction between the C and B domains of EII^{mtl} exists, which is only broken upon unfolding of the C domain. However, at the T_m of the C domain, the unfolding of the B domain has already taken place, and therefore the results can only be explained in two ways: in the presence of mannitol a part of the B domain undergoes an unfolding transition that is strongly coupled to the unfolding of the C domain and therefore cannot be observed in its absence, or the C domain interacts with the unfolded polypeptide chain from the B domain. In the latter case, however, it is difficult to understand why the effect is so much larger in the presence of mannitol than in its absence.

On the basis of the large difference of the heat capacity increment of binding to wild type EII^{mtl} and tryp-IIC^{mtl}, determined from titration calorimetry (-4.0 and $-0.5 \text{ kJ K}^{-1} \text{ mol}^{-1}$, respectively), we have recently proposed a model for EII^{mtl} in which the binding of mannitol leads to the formation of a complementary surface between the B and C domains (33). The newly formed interaction could result either from folding of previously unstructured parts of the polypeptide chain or from docking of two existing surfaces in the protein. The large change of the enthalpy of unfolding of the C domain in the wild type enzyme upon the addition of mannitol compared with tryp-IIC^{mtl} from the same preparation seems to suggest the former possibility as the most likely, with the thermal unfolding of the newly formed structure coupled to the partial unfolding of the C domain. This interpretation would also explain the very low enthalpy of unfolding observed for the B domain if it is assumed that the unstructured part is localized in this part of the protein. Unfortunately, the quality of the data and the small enthalpy of unfolding of the B domain do not allow us to accurately assess the effects of the binding reaction on this transition.

The results obtained in the presence of perseitol differ considerably from those obtained after the addition of mannitol. In general, the effects are much smaller; an increase of $0.5\text{--}1^\circ\text{C}$ for T_m and $6\text{--}12\%$ for ΔH for the wild type enzyme are observed. In fact, these differences are so small that we cannot be sure whether perseitol is still bound at the melting temperature of the C domain, although binding of perseitol at room temperature was confirmed by flow dialysis in a competition experiment with radioactively labeled mannitol (data not shown). So despite the fact that the thermodynamics of binding of the two compounds, as measured in inside-out membrane vesicles by titration calorimetry, are almost identical (33), the binding of mannitol leads to a much larger shift in T_m and ΔH than the binding of perseitol. It therefore appears that the binding proceeds in two steps, with the second, slow, step leading to a stabilization of the complex and the change in T_m and ΔH and only occurring in the case of mannitol. Indeed such a two-step mechanism has been proposed from an analysis of the binding kinetics of mannitol and perseitol (41), the second step being a conformational change to an occluded state in which mannitol is not accessible from either side of the membrane. It was also proposed that the change to the occluded state cannot take place when perseitol is bound, presumably

because of steric repulsion with the C-7 moiety of this ligand (34).

Interactions between the B and C domains of EII^{mtl} have been demonstrated in a number of studies. Lolkema *et al.* (12) reported that the mannitol translocation rate catalyzed by the C domain increases by 3 orders of magnitude upon phosphorylation of the B domain, while Boer *et al.* (13) showed a dependence of the kinetics of mannitol binding on the nature of the side chain at position 384, normally the cysteine at the active site of the B domain. These data are in agreement with our findings here and support the proposed model. A definitive answer to questions regarding the mechanism of substrate binding and transport, however, awaits the elucidation of the structure of this enzyme in its substrate bound and free state.

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